# Metabolism of Carbaryl by a Soil Fungus

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The fungus *Gliocladium roseum*, which was isolated from soil, metabolized carbaryl (1-naphthyl *N*-methylcarbamate) to three metabolites which were isolated by thin-layer chromatography. They were identified as 1-naphthyl *N*-hydroxymethylcarbamate, 4-hydroxy-1-naphthyl methylcarbamate, and 5-hydroxy-1-naphthyl methylcarbamate by ultraviolet, infrared, and mass spectroscopy. This proves that *N*-alkyl- and aromatic ring-hydroxylation of carbaryl are important detoxication reactions of the investigated fungus. The decrease of radioactivity from the growth medium containing side-chain labeled carbaryl indicated also that a further degradation of the formed metabolites occurs, or that an additional pathway is involved in carbaryl metabolism.

The biological decomposition of carbaryl (1-naphthyl *N*-methylcarbamate), an important insecticide, can occur by hydrolysis of the carbamate ester group followed by degradation of the hydrolyzed fragments (Casida, 1963), hydroxylation of the insecticide molecule (Baron *et al.*, 1969; Dorough *et al.*, 1963; Oonnithan and Casida, 1968), or conjugation of the original or transformed molecule (Knaak *et al.*, 1968; Kuhr and Casida, 1967; Paulson *et al.*, 1970). All these observations were performed *in vivo* with plants, insects, mammals, or with *in vitro* studies of enzyme systems from insects and mammals. There is only one report (Bollag and Liu, 1970) which describes carbaryl decomposition by specific soil microorganisms, although early field observations indicated that the microbial transformation was a major factor in the degradation of carbamates (Freed, 1951).

The present study was undertaken to evaluate *Gliocladium roseum*, a fungus isolated from soil, with respect to its ability to degrade carbaryl.

## MATERIALS AND METHODS

A carbaryl-degrading fungus, identified as *Gliocladium roseum* (Link) Thom (Gilman, 1957), was isolated from soil which had been treated with the insecticide for a period of 4 weeks. Isolation of the fungus was made by dilution plating technique on mycological agar (Difco) containing 100 ppm of streptomycin (Pfizer) and 100 ppm of penicillin (Pfizer).

The analyses for carbaryl decomposition were made in an enriched medium containing 0.01 $\mu$Ci of methyl-$C^{14}$ labeled carbaryl; carbaryl, 0.1; nutrient broth (Difco), 8 g; yeast extract, 1 g; dextrose, 10 g; $NaH_2PO_4 \cdot H_2O$, 6 g; and $Na_2HPO_4$, 0.5 g in 1 l. distilled water (final pH 6.0). Carbaryl was sterilized by membrane filtration (0.22 $\mu$ pore size Millipore filter) and added aseptically to the autoclaved media. The culture solution was inoculated with spore suspension harvested from mycological agar slants and incubated on a rotary shaker (250 oscillations/min) at 28° C. Each treatment included triplicates and each experiment was repeated twice. For isolation of carbaryl metabolites, *Gliocladium* was grown in a 2-l. Erlenmeyer flask containing 1.5 l. of the medium and incubated on a rotary shaker for 7 days. The culture filtrate was freeze-dried to 5% of its volume and extracted twice with double volumes of ether. Subsequently, the extract was reduced approximately 20 times by evaporation.

Thin-layer chromatography was used to separate carbaryl and its metabolic products. For routine analysis, 20 × 20 cm glass plates coated with silica gel G, F-254 of 250 $\mu$ thickness (Brinkmann Instruments Inc., N.Y.) were employed; however, for preparative scale tlc, the coatings were 0.5 mm thick. The chromatograms were developed with ether-hexane (4:1, v/v) or with methylene chloride-acetonitrile (4:1, v/v) for better separation of the metabolites. Subsequently, the plates were sprayed with a 15% potassium hydroxide solution, air dried, sprayed with 1 *M* acetic acid in methanol, and 0.1% methanolic *p*-nitrobenzenediazonium fluoborate. If metabolites had to be recovered, the plates were not hydrolyzed or visualized. Each isolated compound was purified twice by tlc and then recrystallized from anhydrous ether. For serial analysis, a colorimetric method using *p*-nitrobenzenediazonium fluoborate as chromogenic reagent was applied (Miskus *et al.*, 1959).

Radioactive compounds on thin-layer plates were measured by scraping the entire spot into a scintillation solution containing 4 g Omnifluor and 40 g Cab-O-Sil (Thixotropic suspension powder) in 1 l. of toluene. Aqueous samples were counted in a Bray solution using a 314 E Packard Tri-Carb liquid scintillation spectrometer.

Infrared spectra of the metabolites were taken with a Model 621 Perkin-Elmer spectrometer using a micro KBr disk technique, and ultraviolet spectra were obtained with a Bausch and Lomb Spectronic 505 spectrometer. Mass spectra were taken with a Model 902 mass spectrometer (Associated Electrical Industries, Ltd., England).

Carbaryl, 1-naphthyl *N*-methyl (Carbamate-$C^{14}$), with a specific activity of 26.4 mCi/mmole was purchased from Nuclear

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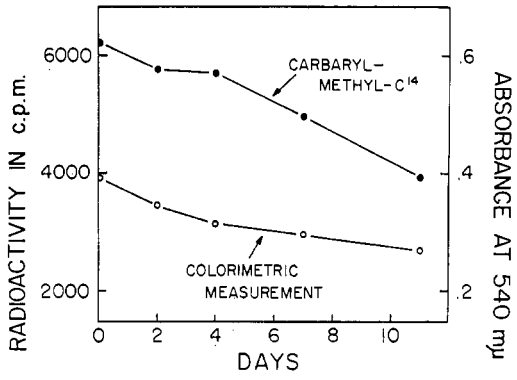


**Figure 1. Decrease of carbaryl in the growth medium as indicated by radioactive and colorimetric measurements**

Chicago Corp., Ill. Carbaryl, 1-naphthyl *N*-hydroxymethylcarbamate, 4-hydroxy-, and 5-hydroxy-1-naphthyl methylcarbamate were provided by R. O. Mumma and the Union Carbide Corp.

## RESULTS

At different growth periods, samples were removed from the fungal growth medium and the decomposition of carbaryl was estimated by radioassay, colorimetric analysis, and thin-layer chromatography. The decrease in radioactivity and colorimetric measurements indicated that partial degradation of carbaryl occurred in the pure culture of the fungus (Figure 1).

Carbaryl and various metabolites were clearly separated by thin-layer chromatography and distinguished also by various color reactions. In addition to carbaryl ($R_f$ 0.90), three other spots were detected with $R_f$ values of 0.50, 0.58, and 0.66, which will be designated as metabolites A, B, and C, respectively. The three metabolites carried radiocarbon, and have the C—O—C(O)—N—C moiety intact. During growth, a gradual formation of the metabolites was observed and varying amounts of radioactive metabolites were found at different time intervals (Table I). From a week old culture, approximately 16.6% of the radioactivity was recovered as metabolites and 83.4% as carbaryl from thin-layer analysis. Metabolites B and C reached their maximum formation after 7 days,

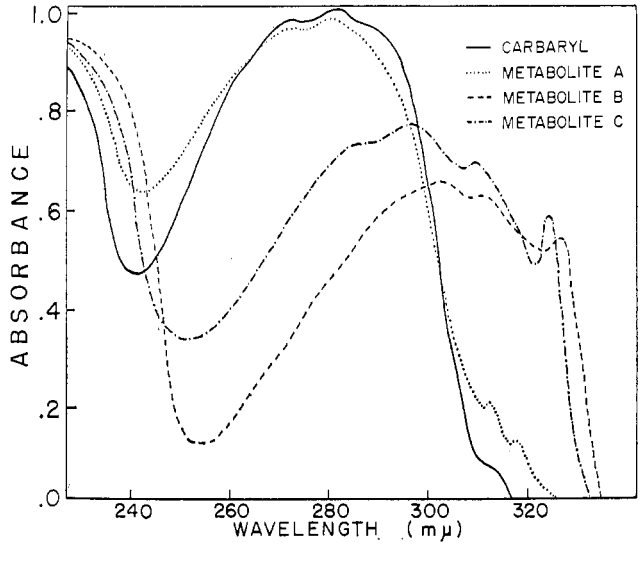


**Figure 2. Ultraviolet absorption spectra of carbaryl and metabolites A, B, and C**

**Table I. Distribution of Radioactivity as Carbaryl and Metabolites Separated by Thin-Layer Chromatography from the Growth Medium of *Gliocladium roseum* after Different Time Intervals**

| | Time of Incubation | | | | |
|---|---|---|---|---|---|
| | 0 days | 2 days | 4 days | 7 days | 11 days |
| Carbaryl | 11,940[a] | 11,580 | 9886 | 7514 | 6408 |
| Metabolite A | 0 | 0 | 240 | 410 | 450 |
| Metabolite B | 0 | 0 | 184 | 360 | 210 |
| Metabolite C | 0 | 180 | 590 | 696 | 512 |

[a] Radioactivity expressed as cpm.

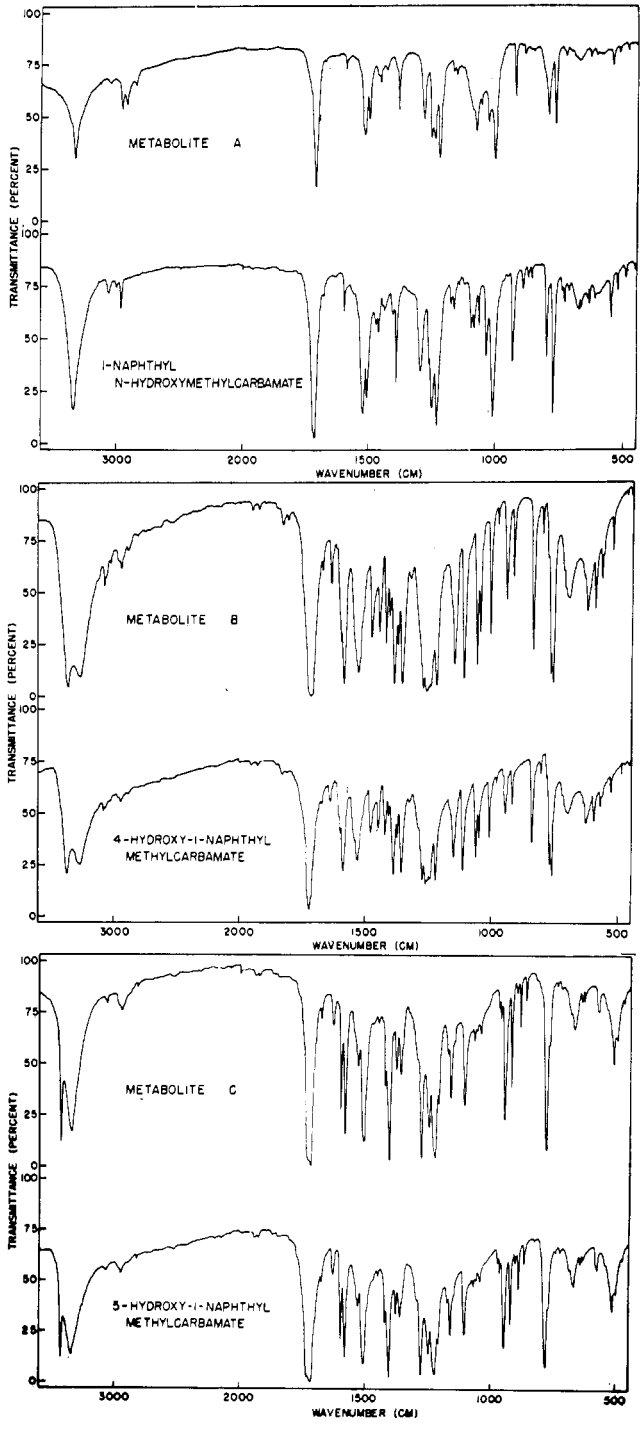


**Figure 3. Infrared spectra of the metabolites A, B, and C and the corresponding authentic chemicals**

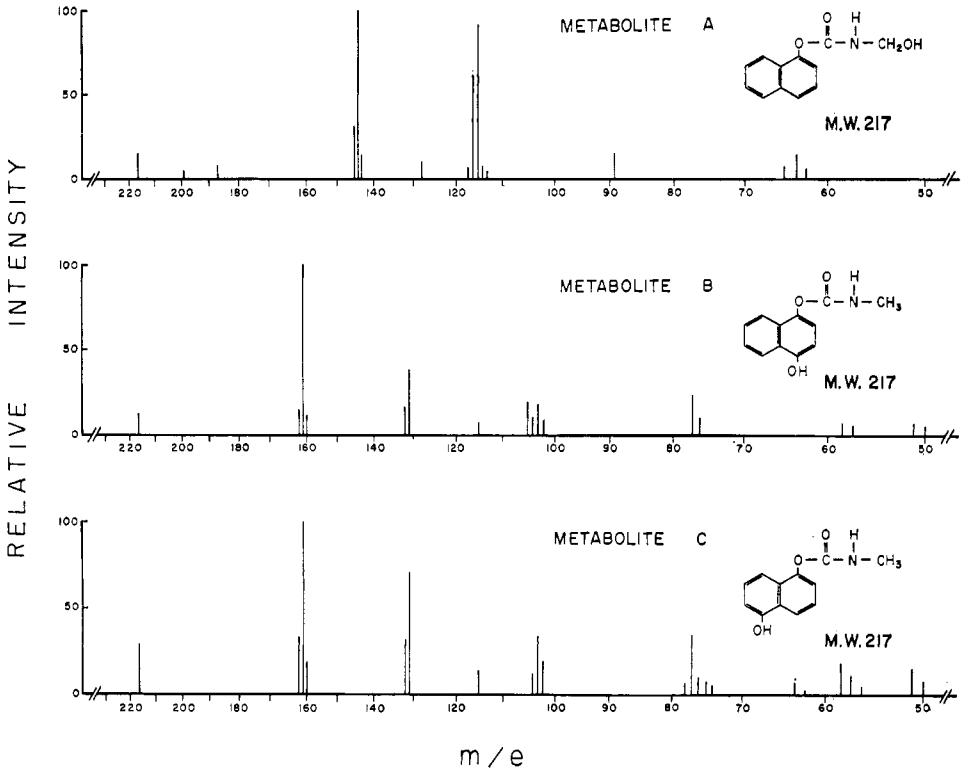


**Figure 4. Mass spectra of metabolites A, B, and C**

but metabolite A reached its maximum formation only after 11 days.

The three radio-labeled metabolites were tentatively characterized by cochromatography with the respective authentic samples. These metabolites absorbed ultraviolet light on tlc and gave a color reaction to the fluoborate reagent. Metabolite A showed a color reaction similar to that of carbaryl, and its $R_f$ value corresponded to that of 1-naphthyl *N*-hydroxymethylcarbamate. Metabolite B had the same $R_f$ value as 4-hydroxy-1-naphthyl methylcarbamate and was also visualized as a pink spot; metabolite C was identical to 5-hydroxy-1-naphthyl methylcarbamate in $R_f$ value as well as in its violet color reaction.

The ultraviolet spectrum of metabolite A was essentially the same as that of carbaryl itself, with a major peak at 282 mμ in ether. Metabolite B gave the uv spectrum identical to 4-hydroxy-1-naphthyl methylcarbamate with a maximum at 303 mμ, and metabolite C was similar to that of 5-hydroxy-1-naphthyl methylcarbamate with a maximum at 298 mμ (Figure 2).

Further identification of the three metabolites was achieved by infrared spectrometry. Comparison of the infrared spectra of the isolated metabolites with those of authentic compounds proved their identity (Figure 3).

Mass spectral analyses confirmed also the molecular weight of the three metabolites which were consistent with the structure of the respective known compounds (Figure 4). The fragment patterns of the ring-hydroxylated metabolites were very similar. The molecular weights of these metabolites as indicated by the mass spectra data were shown to be 217 ± 0. The peak corresponding to hydroxynaphthalene (*m/e* 160) was observed with both metabolites, and the consecutive losses of CO and COH from the parent ion gave rise to *m/e* 132 and 131 fragments. The mass spectral data of metabolite A established the molecular weight as 217 ± 0, which corresponds to that of 1-naphthyl *N*-hydroxymethylcarbamate. The losses of $H_2O$ and $CH_2O$ from the parent ion were observed to form peaks at *m/e* 199 and 187. The latter peak corresponds to 1-naphthyl carbamate. In subsequent fragmentations, the loss of $HOCH_2NCO$ resulted in a 1-naphthol peak (*m/e* 144), and the later losses of CO and COH from the 1-naphthol ion gave peaks at *m/e* 116 and *m/e* /115.

On tlc, 1-naphthol, the hydrolysis product of carbaryl, has not been detected from the *Gliocladium* culture solution, yet a spot appeared in the position of 1-naphthol from the control medium. This phenomenon indicated that the fungus was active in degrading 1-naphthol, which was probably formed by chemical or even biological hydrolysis of carbaryl.

## DISCUSSION

The present study determined that carbaryl was metabolized by the fungus *Gliocladium roseum*. It was found that hydroxylation of the side-chain yielding 1-naphthyl *N*-hydroxymethylcarbamate or hydroxylation of the aromatic ring yielding 4-hydroxy-1-naphthyl methylcarbamate and 5-hydroxy-1-naphthyl methylcarbamate were important detoxication reactions. The three metabolites had the C—O—C(O)—N—C skeleton intact and are identical to those formed in plants, insects, and mammals. Since the radiocarbon in the side-chain of carbaryl does not disappear with the formation of the hydroxylation products, its partial loss from the growth medium could be caused by further breakdown of the metabolites or by a hydrolytic mechanism in which the carbamate moiety is attacked. Previous work from this laboratory (Bollag and Liu, 1970) gave evidence for a hydrolytic metabolism of carbaryl by several microbes isolated from soil, but no indication of a nonhydrolytic pathway was found.

Oonnithan and Casida (1968) concluded that the great variety of metabolic products encountered with carbamate insecticides results, in part, from the resistance of the methylcarbamate moiety to enzymatic hydrolysis.

The amount of radiocarbon of the three metabolites after thin-layer chromatography separation is different, indicating unequal formation of the various products.

It was also observed that the formation of metabolites can be influenced by changing growth conditions of the fungus; heavy spore suspension used as inoculum or a different nutrient medium seems to promote the formation of specific metabolites.

The degradation products of carbaryl by *Gliocladium* are all less active as anticholinesterase agents than carbaryl itself (Oonnithan and Casida, 1968), but their toxicity to soil microbes needs to be evaluated.

The extent to which carbaryl is hydrolyzed (Bollag and Liu, 1970) or hydroxylated by soil microorganisms seems to be different with individual species, and this question requires further investigation in order to clarify the formation of the predominant metabolites from carbaryl in soil.

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